# INFLUENCE OF SELECTIVE LIGHT SCATTERING ON MEASUREMENTS OF ABSORPTION SPECTRA OF CHLORELLA[1,2]

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Experimental information about the light scattering characteristics of biological cells is surprisingly scarce. Even though all measurements of absorption spectra of intact biological structures must be influenced to some extent by scattering, there have been few investigations dealing specifically with scattering by cell suspensions. However, recent experiments have revealed that light absorbing cells and cellular components in suspension scatter light with a strong spectral selectivity (5, 6). Scattering maxima occur on the long wave length sides at absorption maxima. Other studies have demonstrated that this selective scattering can influence measurements of absorption spectra, shifting apparent band positions by 10 to 15 mμ (7, 8). The present paper describes further investigations of phenomena related to selective scattering. We attempted to obtain information about light scattered at small angles to an incident beam from suspensions of *Chlorella pyrenoidosa* Chick (Emerson strain) by using special experimental methods to measure absorption spectra.

We have made use of the fact that the effects of scattering on measurements of absorption spectra of turbid cell suspensions depend on some of the experimental conditions. Specifically, we measured two series of absorption curves in which a single factor that influences the effects of scattering was varied throughout a series. In the first case, absorption curves of a cell suspension were measured with a special spectrophotometer which allowed the detector to view only that light coming from the cell suspension at certain predetermined angles to the incident beam. In the second series of measurements, we fixed the geometry of the spectrophotometer so that the light detector received only directly transmitted light (parallel to the incident beam) and varied the index of refraction of the suspension medium from curve to curve (see Barer (1)).

## APPARATUS AND PROCEDURE

In order to measure absorption curves by allowing the light detector to observe only light coming from the suspension at specified angles, we constructed a special spectrophotometer by modifying the fluorescence spectrophotometer of French (4). The sample holder and mirror system was replaced by a tungsten light source, and baffles and a suspension vessel were inserted between the exit slit and the light detector (Photomultiplier tube, R.C.A. 6217) (see fig 1). The baffles isolated a parallel (± 1°) beam of light from the monochromator output. A thin (1/8 inch) layer of suspension was used to reduce uncertainties in the angle of observation. A special baffle (angle selecting assembly) was constructed which alternately permitted the photomultiplier tube to observe 0° (directly transmitted) light (through the small center opening) or light scattered at an angle to the incident beam (through the doughnut shaped opening, the center of which was in line with the incident beam). The angle of observation could be increased by moving the angle selecting assembly (and the photomultiplier tube and diffusing plate) closer to the suspension vessel. The doughnut shaped opening was used instead of a simple small hole for defining the angle of observation at non-zero angles since it allowed more light to pass with the same angular resolution. The diffusing plate (made of opal glass) eliminated errors which might be produced by differences in the sensitivity of different parts of the photocathode.

As a control, we also measured absorption curves of the suspensions by placing diffusing plates at the suspension and blank vessels as suggested by Shibata (11). Since the effects of scattering on this latter curve were the least, and since this method has been found to yield absorption curves in general agreement with those obtained by the more reliable integrating sphere technique, which further reduces the effects of scattering (compare curves in references (5) and (7)), we assume that absorption curves measured in this way are good approximations of the actual absorption spectrum of the cell suspensions. For these measurements, the angle selecting assembly was removed, the diffusing plate was placed against the vessels, and the photomultiplier tube was moved to the left.

To determine the spectral absorption curves, we measured separately (using the automatic sweep mechanism) spectral curves of the light passing through the suspension (at a specified angle) and through the blank vessel (at 0°). Actually 1 curve was: (output of monochromator) × (sensitivity of light detector system) × (transmission of suspension) = $f(\lambda)$, while the other was: (output . . . .) × (sensi-

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tivity . . . .) × (transmission of blank) = f'(λ). Since the first 2 terms in the products are the same (see below), "absorbance" curves were obtained by dividing the levels of the curves at 5 mμ intervals and changing to logarithmic coordinates (absorbance = log $I_0/I$).

The 2 types of curves were measured alternately, 3 sweeps of the spectral region investigated being made in each case. The averages of the 3 curves of each type were used to calculate the curves shown in figure 3.

This method requires that the product, (output of monochromator) × (sensitivity of detector system), remain constant during the entire determination of 1 curve. This condition was usually fulfilled as evidenced by agreement between different curves of the same quantity taken at different times. When such curves disagreed, the data were discarded.

Absorption curves of cells in suspension media of different indices of refraction were measured with a Beckman DK-2 recording spectrophotometer. The normal arrangement of its optical system was used to measure directly transmitted light (0°±3°) (arrangement (a) in fig 2), while diffusing plates were placed after the suspension and blank vessels (against the vessel holder) for the 0° to 90° curves (arrangement (c) in fig 2). Further details of this method are given in references (7) and especially (8). Media of different indices of refraction were prepared by dissolving bovine serum albumen (Armour's fraction V) or glycerol in water (buffer). Cells from the same culture were added to each medium to yield suspensions of equal cell density.

## Experimental Results

Dependence of Absorption Spectra on Geometry of Optical System: Experimental absorption curves in which the angle of observation of the light detector was varied are shown in figure 3. The upper curves are absorption spectra measured with the photomultiplier tube observing only light coming from the suspension at 0°, 3.4°, and 32° respectively. The curves are quite different, not only in shape but also in the position of the "absorption" maximum (688 mμ at 0°, 682 mμ at 3.4° and 668 mμ at 32°). Other measurements show this shift to be a gradual one with angle. Although we were not able to extend the measurements to larger angles with this apparatus, the results of our previous measurements of light scattered at 90° are very nearly comparable (5, 6).

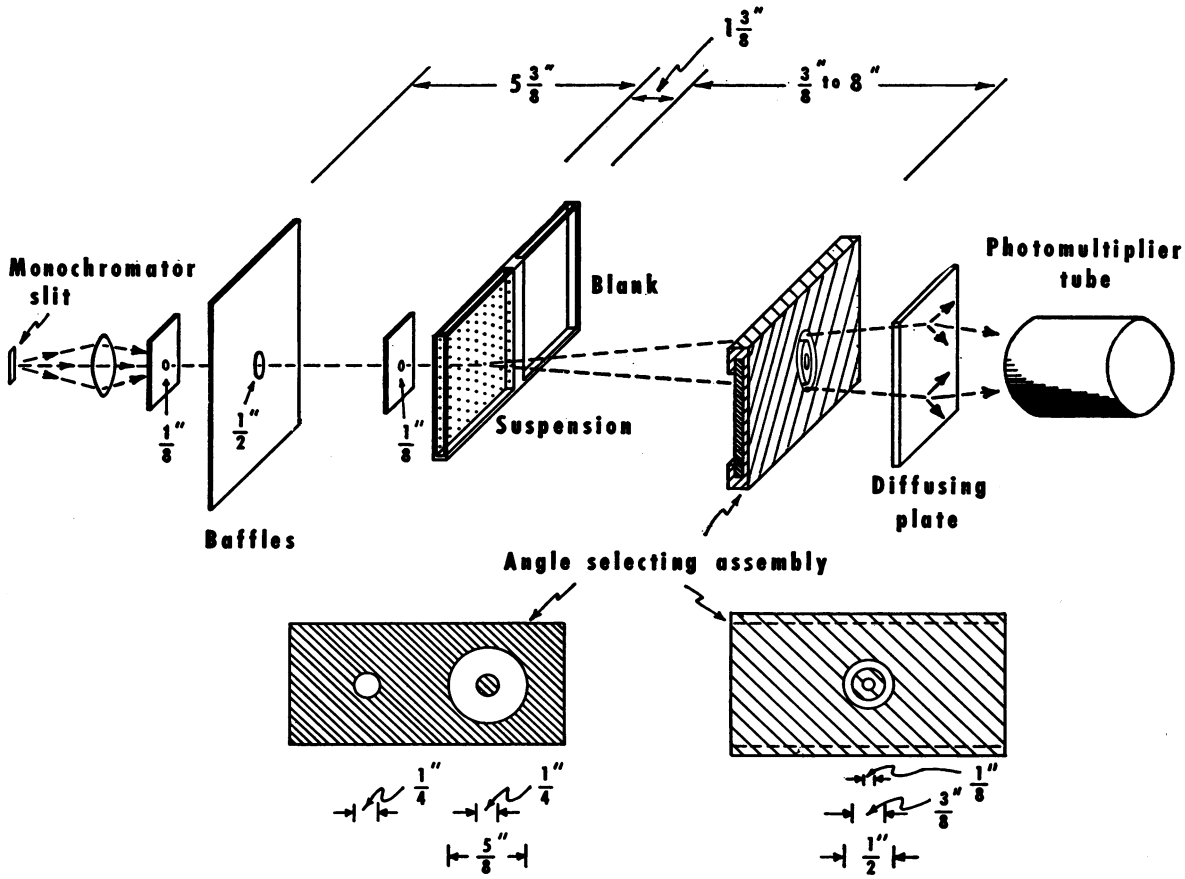



Fig. 1. Apparatus for measuring spectral curves of light transmitted or scattered at small angles to the incident beam. The angle is varied by changing the distance between the suspension and the angle selecting assembly.

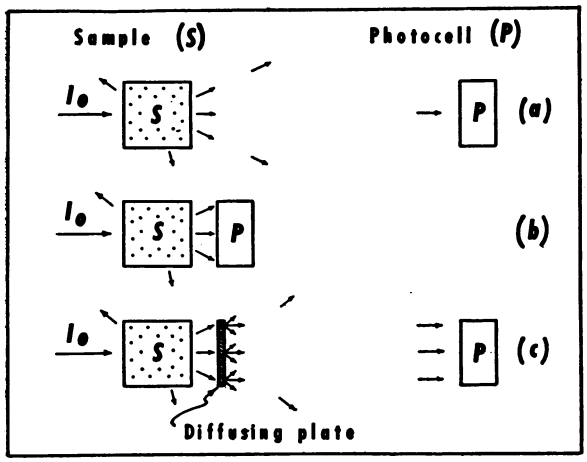



FIG. 2. Schematic diagrams of part of the optical system of a spectrophotometer.

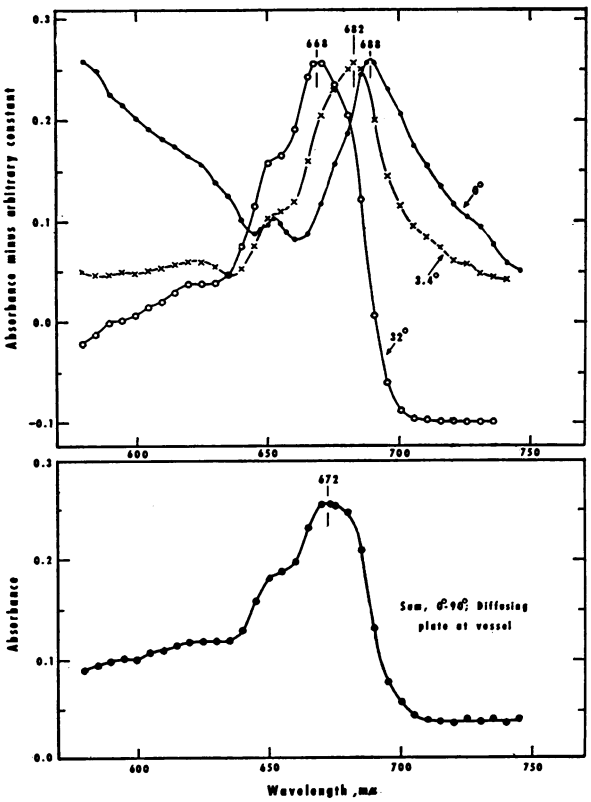



FIG. 3. Chlorella: upper curves, absorption spectra determined by measuring light transmitted or scattered by a cell suspension at indicated angles with the apparatus in figure 1. The curves were arbitrarily normalized in height. The indicated angles, 0°, 3.4°, and 32°, are not corrected for refraction at the vessel surface. This correction changes the angles to 0°, 2.6°, and 24° respectively. The lower curve was determined with the diffusing plate against the vessel, as in Figure 2 (c).

If the curve for 90° scattering by Chlorella were plotted as an absorption curve (this would require inverting the curve and changing from linear to log units), the "absorption" maximum would appear at about 655 mμ which is still further to the left.

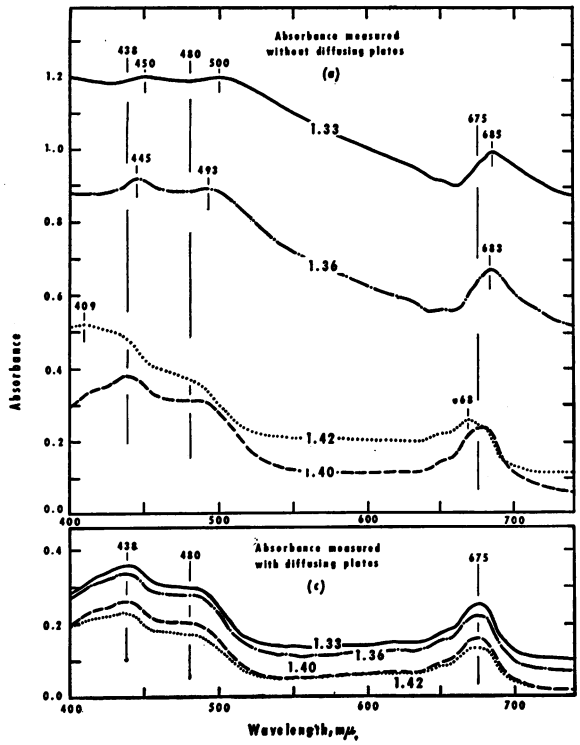
The lower curve in figure 3 (maximum at about 672 mμ) was obtained by placing the diffusing plate at the suspension vessel. It essentially represents a weighted average of spectral curves (such as those shown above it) for light coming out of the suspension at all angles of from 0° to 90° to the incident beam and with absorption maxima varying in position from 688 mμ to about 655 mμ.

DEPENDENCE OF ABSORPTION SPECTRUM ON INDEX OF REFRACTION OF SUSPENSION MEDIUM: Absorption spectra of Chlorella in media of different indices of refraction as measured with the Beckman DK-2 spectrophotometer (using arrangements (a) and (c) in fig 2) are shown in figures 3 and 4. First, it should be noted that the positions of the absorption bands of all suspensions (except that for medium, n = 1.40) are different in the upper and lower series of curves (see reference (7) for a preliminary report of this effect). For instance, the red absorption maximum of cells in water (n = 1.33) is at 685 mμ in the (a) series of curves while it occurs at 675 mμ in the (c) series.

As the index of refraction of the medium is increased towards approximately that of the cells, assumed here to be 1.40, not only does the general height of the curves decrease, but the apparent positions of the bands on the upper curves shifts towards the actual position of the bands. On further increasing the index of refraction of the medium to 1.42 (approximately 40 % albumen), the shift to the left of the band positions continues so that the red band now appears at 668 mμ.

It should be noted that the positions of the bands on the lower curves in figure 4, which presumably represent the best approximation of the actual absorption spectra, are not significantly altered by the medium. Therefore, the variations in the positions of the bands in the upper part of the figure must be attributed to optical phenomena and not to actual differences in the true absorption spectra of the cells.

On the other hand, a similar series of measurements on Chlorella cells in glycerol-water solutions gave quite different results (see fig 5). While the general level of the upper curves is reduced somewhat as the index of refraction of the medium increases, only a slight shift in the apparent positions of the bands is observed. Other curves in the same series for intermediate values of n were similar to those shown in the figure. The general difference between the behavior of the curves in figures 4 and 5 can be attributed to differences in the nature of the additives, bovine serum albumen and glycerol, respectively. The albumen, being a large molecule, does not penetrate the cell membrane, and the osmotic pressure of its solutions is



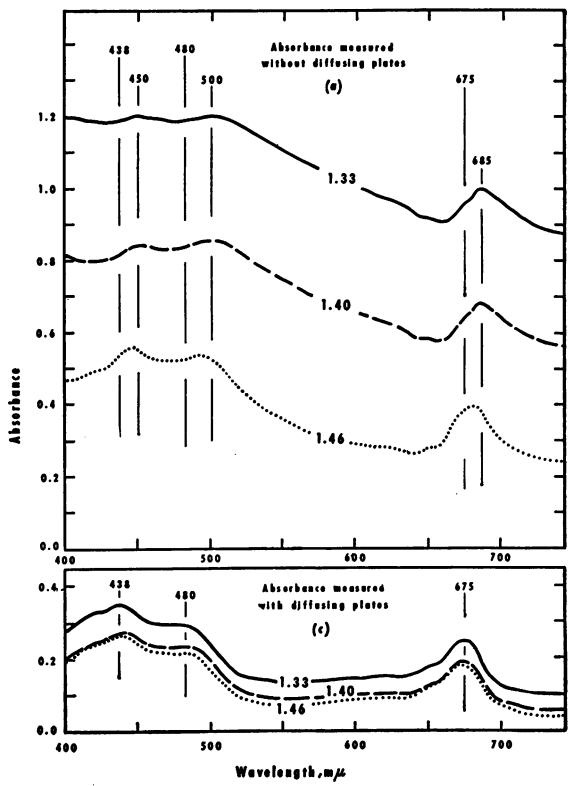




FIG. 4 (*left*). Chlorella: absorption spectra of suspensions of equal cell density in different water solutions of bovine serum albumen having the indicated refractive indices. The curves were measured with a Beckman DK-2 recording spectrophotometer using arrangements (a) and (c) in figure 2.

FIG. 5 (*right*). Chlorella: absorption spectra of suspensions of equal cell density of water-glycerol solutions having the indicated refractive indices.

probably not very different from that of water. However, the small glycerol molecule readily penetrates the cell membrane and the osmotic pressure of its solutions is high. Hence, the optical properties of the cells in glycerol-water solutions must be different from those of cells in water or albumen solutions because of penetration of the glycerol and the exit of water from the cell. Both processes increase the index of refraction of the cells. Scattering depends on the difference between the index of refraction of the cells and that of the surrounding medium (see next section). It is suggested that the addition of glycerol to the medium did not effect large changes in this difference since both indices are raised by it.

As noted elsewhere (8), the positions of the maxima on curves measured with arrangement (a), figure 2, vary slightly with suspension concentration (presumably because some of the observed light has encountered 2 or more cells). For cells in water, the thinnest suspensions gave peaks displaced furtherest to the right. We estimate that on extrapolating to zero concentration, the band maximum at 685 mμ in the upper part of figure 4 would be shifted to about 689 mμ.

## THEORETICAL INTERPRETATION

Many similarities exist between the angular dependent curves in figure 3 and the medium dependent curves in figure 4. In both cases, the position of the "absorption" maximum is found on either side of the actual maximum depending on experimental conditions. Both sets of results are clearly related to the phenomena of selective scattering which can be explained in terms of the theory of scattering by absorbing particles (9).

To a first approximation, total scattering by both absorbing and non-absorbing particles depends on the index of refraction (relative to the surrounding medium) of the scattering particle according to the following equation:

$$S = k\,(m-1)^2 \qquad (1)$$

where S is scattering per unit incident light, k is a constant and m is the relative index of refraction of the particle. (If the index of refraction of cells is 1.40 and that of the medium (water) is 1.33, the relative index of refraction of cells is 1.40/1.33 = 1.05.) According to the theory of anomalous dispersion, m

becomes a complex number in regions of absorption ($m = n - in'$ where we call n the real part and n′ the imaginary part). Both parts vary rapidly and in a characteristic manner with wave length. The imaginary part of m is very nearly proportional to absorption while the real part varies assymetrically about the absorption band. (See figure 6 A for theoretical curves of these 2 parts for Chlorella.) Since the real part of m passes through a maximum on the long wave side of the absorption band, S (in equation 1) should also pass through a maximum on the long wave length side if $m > 1$. This effect which we called selective scattering, was observed in studies of 90° scattering of several different biological systems.

It would be desirable in this section to use an exact theory of scattering by colored particles to calculate theoretical curves for comparison with all of our experimental curves. While it appears that the Mie theory of scattering by light absorbing particles should be capable of explaining all of our results, the calculations for absorbing particles of this size would be almost prohibitively complex. On the basis of limited efforts to apply the theory to Chlorella, we estimate that the determination of a single point on one spectral curve would involve several thousand calculations.

On the other hand, van de Hulst (12) has obtained approximate expressions from the rigorous Mie theory and the classical theory of dispersion which predict extinction and absorption by some homogeneous spheres. The equations, as restated in reference (9), are useful in predicting extinction and total scattering by nearly spherical biological cells. We used it here, in a further modified form which eliminates some of the approximations, to calculate extinction curves for Chlorella cells in media of different indices of refraction. Since the upper curves of figure 4, for Chlorella in bovine serum albumen solutions, are actually extinction curves (extinction = absorption + total scattering), the theoretical curves should agree reasonably well with these experimental curves if, indeed the theory is applicable to suspensions of Chlorella cells.

A Chlorella cell was assumed to be a homogeneous sphere, diameter: 3.2 μ. We assumed its average index of refraction at wave lengths remote from absorption bands to be 1.40. The index in regions of absorption was calculated from the classical theory of dispersion using the extinction coefficient of cells (see reference (9)) and a band half-width of the red absorption band of 33 mμ. The absorption curve was assumed to be symmetrical about 675 mμ. No allowance was made for the shoulder at 650 mμ which is presumably caused by chlorophyll b.

The theoretical extinction curves are shown in

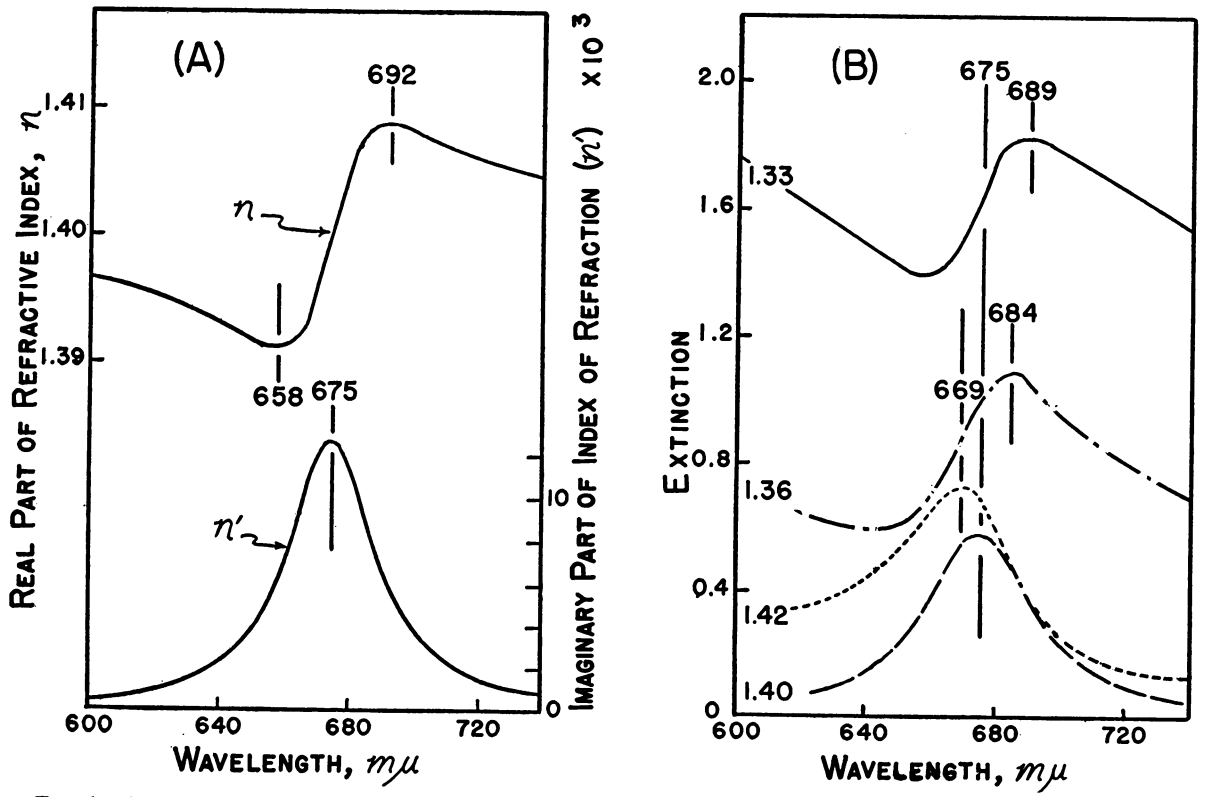



FIG. 6. Chlorella: theoretical curves. (A) Real (n) and imaginary (n′) parts of average index of refraction of entire cell (2). (B) Extinction of cells in media of indicated refractive indices, calculated using (A) above and (12).

figure 6 B. Comparison with the corresponding experimental curves in the upper part of figure 4 reveals that the observed dependence of the band positions on the index of refraction of the medium is clearly predicted by theory. The bands on the theoretical curves at 689, 684, 675, and 669 mμ are in good agreement with those on the corresponding experimental curves at 685, 683, 677, and 668 mμ respectively. It is likely that the discrepancy between band positions, 689 and 685 mμ for cells in medium, n = 1.33, is caused by effects of multiple scattering on the experimental curve as previously mentioned. The difference between the band positions of 677 and 675 mμ for cells in a medium of n = 1.40 may be caused by our assuming for the theoretical calculations, a value (1.40) of the index of refraction of the cells (at wave lengths remote from the absorption band) which is too low.

On the other hand, the general levels of the experimental curves vary more with the index of refraction of the medium than do those of the theoretical curves. This difference between experiment and theory may be caused by the following factors. The logarithmic ordinate scale was used for the experimental curves while the scale for the theoretical curves is linear. Furthermore, thus far we have made no allowance for effects of scattering by non-absorbing components of the cells. While it is difficult to say how the omission of this factor would influence the results, it might easily produce the above mentioned discrepancy between experiment and theory.

There is also another less rigorous, but more tangible, theoretical explanation of the upper curves in figure 4. It might be suggested that the displacement of the absorption band of Chlorella in water (when measured at 0°) from its actual position at 675 to 685 mμ can be explained in terms of the fact that we actually measured extinction which is the sum of absorption plus total scattering. Thus if the actual absorption band lies at 675 mμ and the scattering band at 690 mμ (see reference (5)), a suitable additive combination of the 2 bands would yield an extinction band at 685 mμ. As one increases the index of refraction of the medium (to 1.36 and 1.40) toward that of the cell (about 1.40), the magnitude of scattering (see equation 1) and its contribution to the extinction band is reduced, and the band is shifted from 685 to 683 mμ to about 675 mμ. Now, passing on to a medium (n = 1.42) of index of refraction greater than that of the cells, we see that (m−1) in equation 1 becomes a negative number (although $(m-1)^2$ will nevertheless be positive). In this case, the index of refraction of the cells (see fig 6 A) has a minimum on the short wave length side of the absorption band, so that the quantity (m−1) has a minimum value since m < 1 but nevertheless $(m-1)^2$ has a maximum value. In this case there should be a scattering maximum on the short wave length side of the band instead of the long wave length side. Thus the extinction would be the sum of the absorption (maximum at 675 mμ) and scattering (peak between about 659 and 675 mμ). And indeed, the maximum extinction does seem to represent a sum of 2 such components with a maximum at 668 mμ.

On the other hand, if we consider the upper curves in figure 3 for the angular dependence of the absorption, neither equation 1, van de Hulst's theory, or the above considerations are applicable, and no theoretical explanation is offered at this time. However, some understanding of the observed phenomena of angular dependence of band position may be obtained by considering that advanced by Lothian and Lewis (10) to explain the results of their spectral curves of red blood cells. These authors reported interesting extinction and absorption curves for red blood cells which were measured by methods similar to both of ours for Chlorella in water, n = 1.33 in figure 3. (Their spectral curves have been substantially reproduced under special conditions in our laboratory by J. A. McClure.) While their data might in part be interpreted in terms of a peak shift caused by selective scattering as we suggested above, they explained the differences in their curves in terms of interference and diffraction effects. The explanation involves interference between light passing through the cell with that passing around it.

We have previously described absorption spectra measured with a diffusing plate at the suspension vessel both as reasonable approximations of the actual absorption spectra and also as a composite of a number of different spectral curves such as those in figure 3. It doesn't seem immediately obvious how the diffusing plate curves can be these two things at once. However, the explanation appears to lie in the interpretation of the curves in figure 3 in terms of interference and diffraction effects.

## Discussion and Conclusions

By selecting the experimental conditions for measuring absorption spectra of the green alga *Chlorella pyrenoidosa*, we found that the positions of the absorption bands vary with the conditions over rather wide wave length intervals. The positions of band maxima as well as the shapes of the absorption curves are functions of both the index of refraction of the surrounding medium and the geometry of the optical system of the spectrophotometer. These effects are strictly optical in nature and do not reflect actual changes in the absorption spectra of the cells, which according to curves measured with diffusing plates, remained constant through all measurements.

Actually it should be noted that the position of the red absorption band (672 to 675 mμ) as measured with diffusing plates in figures 3 and 4 is slightly different in the 2 cases. The cells used in these 2 measurements were from different cultures. We believe that this difference is a real one although it might be accounted for in other ways. For instance, different spectrophotometers were used for the 2 sets of measurements and there may have been slight calibrational errors. Also, the diffusing plate does not collect all scattered light from the suspension but only that

emerging in the forward direction. The level of curves at 740 mμ where little if any absorption occurs is probably caused by loss of light caused by back scattering. It is possible that differences in the scattering characteristics of the cells from different cultures so effected the back scattered light that the apparent band position was shifted.

It may well be that many of our curves measured in special ways (upper parts of figs 3, 4, and 5) should not actually be called absorption spectra. This illustrates the fact that while absorption spectra of non-scattering dyes in solution have a rigorous operational definition, no counterpart exists for turbid suspensions. However, in normal measurements of absorption spectra of biological cells and structures, we attempt to obtain curves which approximate those which would be obtained if the light absorbing compounds, in the same physical and chemical state, were uniformly distributed in a medium such that no scattering occurred. Unfortunately, these experimental conditions cannot be attained even in an integrating sphere which detects a representative sample of all light scattered out of the suspension vessel. While the integrating sphere appears to provide the most reliable measurements of absorption spectra, results obtained with it, as well as by most other methods, are nevertheless influenced by second order effects of scattering within the suspension (such as alteration of the effective path length of light through the suspension). Another factor which distorts absorption curves is the mutual shading effect which has been described theoretically for non-scattering particulate suspensions by Duysens (3) and E. E. Jacobs (the effect flattens absorption bands).

The optical effects reported here, and also those mentioned above, point to the fact that the events which occur when a beam of light enters a suspension of biological cells are still poorly understood. It appears at the present that instead of attempting to devise, by empirical means, a better experimental method of measuring absorption spectra of cell suspensions, it is more appropriate to first gain a clearer understanding of the events which occur on the passage of light through the suspension. With this, perhaps we may be in a better position to intelligently interpret the results of any spectroscopic measurements on turbid systems.

## Summary

The scattering of light by *Chlorella pyrenoidosa* was investigated by measuring absorption spectra as influenced in different ways by scattering. Two series of measurements were carried out. In one case the cells were suspended in buffer solution and absorption spectra were measured with the light detector observing only that part of the total transmitted or scattered light coming from the suspension at well defined angles. In the other case, the cells were suspended in media of various indices of refraction and absorption spectra were measured by allowing the light detector to observe only directly transmitted light. The apparent positions of the absorption bands were found to be strong functions of both the angle of observation of the light detector and the index of refraction of the medium. The latter results were found to agree well with the predictions of scattering theory.

The vessel and baffle assembly in figure 1 were constructed by Mr. Richard Hart, who also drew most of the figures. Messrs. J. A. McClure and C. W. Hamlet performed some of the calculations. We also wish to thank Drs. C. S. French and J. H. C. Smith and Mr. H. W. Milner for their many helpful suggestions. It is appropriate at this time to point out that our study of light scattering by cell suspensions, which began when the author was a student at the Photosynthesis Project of the University of Illinois, was first suggested by the late Dr. Robert Emerson.

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